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Inhalation of Sidestream Cigarette Smoke Accelerates Development of Arteriosclerotic Plaques

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Background. Environmental tobacco smoke has been blamed for ~40 000 excess deaths from heart disease annually in the United States. As yet, no pathophysiological process that could be responsible for these deaths has been identified. Environmental tobacco smoke is composed mainly of aged and diluted sidestream smoke but also contains 15% to 20% exhaled mainstream smoke. Carcinogens, including nitrosamines and polynuclear aromatic hydrocarbons, are present in mainstream smoke and sidestream smoke. Carcinogen levels in sidestream smoke, unlike those in mainstream smoke, are not reduced in filtered cigarettes. The US Environmental Protection Agency has designated environmental tobacco smoke as a human (class A) carcinogen. In cockerels, subtumorigenic doses of polynuclear aromatic hydrocarbons accelerate aortic arteriosclerotic plaque development.

Methods and Results. To determine whether sidestream smoke inhalation affects arteriosclerotic plaque development, we exposed cockerels to sidestream smoke (n=30) or to filtered air (n=12) in inhalation chambers for 6 hours per day, 5 days a week from 6 to 22 weeks of age (0.4% of projected lifespan). Chamber levels of carbon monoxide, total suspended particulates, and nicotine were measured regularly during the exposures. The abdominal aorta from each cockerel was cut into 10 segments, and the plaque index (mean plaque cross-sectional area [mm²]/mean luminal circumference [mm] × 100) was calculated for each segment. There were no differences in plaque incidence or distribution between sidestream smoke-exposed and control cockerels; however, plaque indexes were significantly greater for sidestream smoke-exposed than control cockerels in all segments.

Conclusions. Thus, relatively brief exposures to sidestream smoke early in life are sufficient to enhance arteriosclerotic plaque development. (*Circulation*. 1993;88[part 1]:1820-1825.)

KEY WORDS • smoking • heart diseases

(*) Cocker,

Cigarette smoking is a major contributing factor to heart disease. In the 1983 US Surgeon General's report,¹ the incidence of coronary heart disease (CHD) was noted as being twice as high for smokers and four times higher for heavy smokers than for nonsmokers. Death rates from CHD were 70% higher for smokers and more than twice as high for heavy smokers than for nonsmokers. In addition to the well-known risks posed to smokers by inhalation of mainstream smoke, there are now more recently perceived risks posed to the health of nonsmokers due to involuntarily inhalation of environmental tobacco smoke.

Environmental tobacco smoke is composed mainly (85%) of aged and diluted sidestream smoke. The remainder is exhaled mainstream smoke. Up to 40 000 excess heart disease deaths yearly have been attributed to environmental tobacco smoke exposures.²⁻⁴ In 1992, the American Heart Association issued a position paper on environmental tobacco smoke and cardiovascular disease.⁵ This document notes that environmental tobacco smoke is a major preventable cause of cardiovas-

cular disease and death; that environmental tobacco smoke should be classified as an environmental poison; and that its removal from home, work, and public environments should be pursued.

There have been only a limited number of in vivo studies designed to identify specific alterations to the cardiovascular system caused by inhalation of environmental tobacco smoke. Most of these have employed acute exposures and have targeted end points only peripherally related to CHD.⁶⁻⁸ Recently, three reports appeared suggesting possible mechanisms whereby environmental tobacco smoke could play a direct role in development of heart disease. In the first of these, sensitization of neutrophils by environmental tobacco smoke was proposed to precede their activation. This, in turn, could result in oxidant-associated tissue injury.⁹ In the second study, increased carotid wall thickness was detected in human volunteers who reported having been exposed routinely to environmental tobacco smoke.¹⁰ This increased wall thickness correlated with increased weekly exposure to environmental tobacco smoke. The results of the third experimental study as reported by Zhu et al¹¹ showed that atherosclerosis increased in male New Zealand White rabbits maintained on a high cholesterol diet and exposed concomitantly to sidestream smoke. In that study, relatively high levels of sidestream smoke were used, as determined by analysis

Received December 21, 1992; revision accepted May 28, 1993.
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of carbon monoxide, total suspended particulate, and nicotine levels. In addition, the diets of the rabbits employed in the study were supplemented so that serum cholesterol levels were doubled. In the study we report, sidestream smoke was used as a surrogate for environmental tobacco smoke. Moderate levels were chosen so that exposure conditions would be more relevant to those encountered in indoor environments by passive smokers. In addition, our animals received a low-fat diet that was not supplemented with cholesterol. In both studies (Reference 11 and the present study), the same pathophysiological end point was selected, namely, arteriosclerotic plaque development. Although the exposure protocols, dietary modification, animal model, and analytical methods used by Zhu et al were all different than the ones we used, the results complement well the ones described here (see "Discussion").

In the animal model we use, the cockerel, fibromuscular arteriosclerotic plaques develop spontaneously in the abdominal aorta and are similar histologically and ultrastructurally to human coronary artery plaques.¹² These spontaneous cockerel plaques are generally microscopic for the first 6 months of life. Environmental agents including viruses,¹³⁻¹⁵ chemical carcinogens,¹⁶⁻¹⁹ and mainstream cigarette smoke²⁰ can induce and/or accelerate vascular pathologies in a number of avian species, including cockerels. Although polynuclear aromatic hydrocarbon carcinogens have long been used as initiating agents in studies of multistage carcinogenesis,²¹ their principal effect in the cockerel, when administered in subtumorigenic doses, is to accelerate the development of preexisting arteriosclerotic plaques.^{16,17,22} Since polynuclear aromatic hydrocarbons carcinogens are present in sidestream smoke,²³ it would be reasonable to assume that sidestream smoke may also accelerate the development of plaques in cockerels.

To address directly the question of whether inhalation of sidestream smoke accelerates aortic plaque development, 30 6-week-old cockerels were exposed in dynamic inhalation chambers²⁴ for 6 hours a day, 5 days a week, for 16 weeks to sidestream smoke generated by the steady-state combustion of five low-moderate tar, filtered, reference (1R4F) cigarettes. Twelve control cockerels in similar inhalation chambers were exposed to filtered, conditioned air. Chamber levels of carbon monoxide, total suspended particulates, and nicotine were measured regularly to provide independent measures of the amount of smoke generated. To determine the effects of the exposures on plaque development, the abdominal aorta from each sidestream smoke-exposed and air control cockerel was cut into 10 5-mm segments, and the plaque index (plaque cross-sectional area [mm²]/luminal circumference [mm] × 100) was calculated for each of the 10 segments. All animals were fed a standard, low-cholesterol diet to minimize confounding factors in plaque development.

Methods

Animals

White leghorn cockerels (Avian Services, Frenchtown, NJ) were received at our facility at 4 weeks of age. Before random distribution into sidestream smoke-exposed and air control groups, animals were quarantined for 2 weeks. During this time, they were acclimated to a 12-hour

light/dark cycle and observed for anomalous behavior and disease. During the study, animals were housed in large stainless-steel cages, and AALAC guidelines were followed for animal housing and care. Food (Chick Starter Grower, Ralston Purina, St Louis, Mo) and water were available ad libitum, except when the cockerels were in the exposure chambers.

Exposures

Exposures were carried out simultaneously in four 1.3-m³ stainless-steel and Plexiglas dynamic exposure chambers.²⁴ Concurrent with the sidestream smoke exposures, 12 age-matched control cockerels, in two adjoining chambers, were exposed to filtered air.

The sidestream smoke was generated by a smoking machine (AMESA Technologies, Geneva, Switzerland), modified to smoke five cigarettes. The smoke generator was positioned within an exposure chamber. Tubing connected to the mainstream smoke ports vented the mainstream smoke to a series of traps outside the smoke-generating chamber. The sidestream smoke-generating chamber was connected at the top via 2-in. pipe to the four adjacent 1.3-m³ chambers in which the cockerels were exposed. The four chambers were balanced so that all received the same amount of sidestream smoke. Airflow of HEPA-filtered air into each of the chambers was maintained at 300 L/min (14 air changes per hour). Air was conditioned so that temperature within each chamber was maintained at 70°C. Relative humidity was ambient. The puff characteristics of the generated smoke were volume of 30 mL; interval, 15 seconds; and duration, 2 seconds. Burning cigarettes, expelled from the generator when the butt length reached 45 mm, were replaced automatically with full-length cigarettes that were lit by the generator's filament.

Concentrations of sidestream smoke in the exposure chambers were monitored by measuring total suspended particulate levels and carbon monoxide levels at 2-hour intervals during the daily 6-hour exposures. Particulate levels were determined gravimetrically after passing measured amounts of exposure air through 0.45- μ m membrane filters (Gelman Sciences Inc, Ann Arbor, Mich). Carbon monoxide levels were determined using a portable carbon monoxide analyzer (Interscan Corp, Chatsworth, Calif). Exposure chamber air was passed through a high-volume 0.2- μ m filter (Gelman) before sampling by the analyzer. Nicotine levels were determined weekly by passing exposure chamber air through tubes containing XAD-4 resin (SKC Inc, Eighty-four, Pa). The tubes were analyzed by Maryland Spectral Services (Baltimore, Md).

Exposure Protocols

Thirty white leghorn cockerels were exposed for 6 hours a day, 5 days a week, for 16 weeks to sidestream smoke produced by the steady-state combustion of five 1R4F reference cigarettes (THRI, University of Kentucky, Lexington, Ky). Twelve cockerels were exposed to filtered, conditioned air in duplicate chambers following the same exposure protocol.

Plaque Analysis

After the cockerels were killed, aortas were cut longitudinally, fixed in phosphate-buffered formalin

TABLE 1. Daily Concentrations of Smoke Parameters During 16-Week Exposure to Sidestream Cigarette Smoke

	Chamber A	Chamber B	Chamber D	Chamber E
Total suspended particulates (mg/m ³)	7.76±1.67	8.02±1.49	7.50±1.60	8.41±1.78
Carbon monoxide (ppm)	35.38±4.86	34.87±4.23	33.62±4.19	35.00±4.61
Nicotine (μg/m ³)	378.5±64.4	383.2±62.1	385.1±79.5	414.3±60.0

Smoke exposures were conducted simultaneously in four identical 1.3-m³ chambers. Total suspended particulates and carbon monoxide were measured 3 times a day. Nicotine was measured weekly. Values are mean±SD.

(pH 7.4), randomized, and sent to a histologist for coding and processing. The abdominal aorta from each cockerel was cut into 10 5-mm segments. Segment 1 was the most distal, and segment 10 was closest to the thoracic aorta. Then, 50-μm-thick cross sections were cut from the distal face of each of these segments. The sections were mounted on slides and stained with the Verhoef-van Gieson stain. The images of the stained sections were projected from a Zeiss Photomicroscope via a Panasonic Digital 5000 Color CCD camera onto a Panasonic color display monitor. All segments were analyzed. For each section, plaque cross-sectional area (if plaque was present) and the luminal circumference were measured directly, in triplicate, with the aid of a Summagraphics model 1601 digitizing tablet and the BioQuant System IV software program. All slides were read double blind. Plaque indexes (mean plaque cross-sectional area [mm²]/mean luminal circumference [mm]×100) were calculated from the means of each triplicate determination for each of the 10 aortic segments from each sidestream smoke-exposed and air control cockerel.

Data Analysis

The plaque index values from both sidestream smoke-exposed and air control groups fit lognormal distributions. For all aortic segments analyzed from both groups, all except for one segment median plaque index value were lower than the corresponding mean plaque index values. Median and mean plaque index values were the same only for segment 1 from the sidestream smoke group (see Fig 2A). This skewing of plaque index values necessitated a transformation of the data for statistical evaluation. The logarithms of the control and sidestream smoke-exposed plaque index values were arranged according to increasing value and plotted on log-probability coordinates. Linear regression lines were calculated via least-squares analysis (Minitab) and drawn for each data set. This approach is independent of plaque segment position. Analysis of covariance was used to test for differences between the two regression lines.²⁵

Results

Mean exposure concentrations of carbon monoxide, total suspended particulates, and nicotine from all four exposure chambers are displayed in Table 1. The slight differences between chambers in the concentrations of these smoke parameters are not physiologically relevant. We had determined previously that there is a linear increase in the quantities of these agents produced as the numbers of cigarettes smoked increases from one to five (Penn and Snyder, unpublished observations).

The results of the plaque analyses are presented in Table 2. Microscopic plaques were present in the abdominal aortas of all except one cockerel (12 of 12 air controls and 29 of 30 sidestream smoke-exposed animals). Table 2 shows that daily exposure of cockerels to sidestream smoke had no significant effect on plaque numbers. There were about 1.5 plaques per cockerel in both air control and sidestream smoke-exposed groups (Table 2A). The mean numbers of plaque-containing segments did not differ significantly in the two groups (Table 2B; Student's *t* test, *P*>.10). Plaque was present in 50 of 120 (41.7%) air control segments and 153 of 300 (51%) sidestream smoke-exposed segments. These distributions are not significantly different (χ^2 test, *P*=.11). In addition, the percentage distribution of plaque within aortic segments was very similar in both groups (Fig 1).

While inhalation of sidestream smoke had no effect on plaque number, it caused a marked increase in plaque size, as determined by plaque index measurements. The latter permit a segment by segment analysis of plaque size as well as segment-independent analyses (see "Data Analysis" in "Methods"). Average plaque index values by segment, for all cockerels, are presented in Fig 2. The plaque index values from 3 to 10 samples in air controls and from 6 to 24 samples in sidestream smoke-exposed animals are represented in each segment. For any given segment, mean plaque index values were greater for sidestream smoke-exposed than for control cockerels (Figs 2A and 2B, solid line).

In the sidestream smoke-exposed group, all except for one segment median plaque index value (1) were lower than the corresponding mean plaque index values (Fig 2A). In the air controls, all segment median plaque index values were lower than the corresponding means (Fig 2B). This skewing of segment plaque index values necessitated a transformation of the data for statistical evaluation. Both sets of data fit lognormal distributions. Previous work with polynuclear aromatic hydrocarbons—

TABLE 2. Number of Plaques and Plaque-Containing Segments per Cockerel

	Air Controls (n=12 Cockerels)	Sidestream Smoke-Exposed (n=30 Cockerels)
Plaques	1.58±0.14*	1.53±0.09*
Plaque-containing segments	4.2±0.7†	5.0±0.4†

*These values are not significantly different from each other. (Student's *t* test: With df=40 and *t*=.0196, *P*>.90.)

†These values are not significantly different from each other. (Student's *t* test: With df=40 and *t*=.091, *P*>.10.)

Values are mean±SEM.

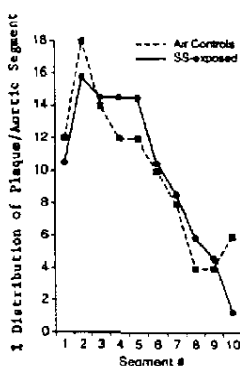


FIG 1. Plot of percentage distribution of arteriosclerotic plaque in each aortic segment of sidestream smoke-exposed (—) and air control (----) cockerels.

treated cockerels also revealed log-normally distributed plaque sizes.¹⁶ The logarithms of the 50 control and 153 sidestream smoke-exposed plaque index values were arranged according to increasing value and plotted on log-probability coordinates (Fig 3). Linear regression lines were calculated via least-squares analysis and drawn for each data set. The linear correlation coefficients for each regression line exceeded .99. Analysis of covariance methods were used to test for differences between the two linear regression lines. This approach formally tests for the differences by comparing the explanatory power of two separate models. The first model assumes that one regression line can be fitted to all of the experimental data, while the second model fits separate lines (ie, with different slopes and intercepts) to the sidestream smoke-exposed and air control groups. With this analysis, there was a highly significant difference between the air control and sidestream smoke exposure regression lines ($F=284$; $P<.0001$). The calculated intersection of the regression lines occurs at a probit value of 2.44. This corresponds to a plaque index value of 0.20, which is 50% lower than the lowest plaque index value that was observed in these studies. Thus, there is no overlap anywhere within the two data sets. It is reasonable to conclude from these results that in this animal model, moderate exposure to sidestream smoke for a brief period early in life is sufficient to markedly accelerate arteriosclerotic plaque development.

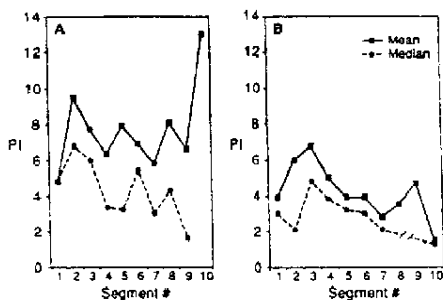


FIG 2. Comparison of mean (—) and median (----) plaque index values per segment for sidestream smoke-exposed (A) and air control (B) cockerels. Note that in all cases, mean values exceed corresponding medians.

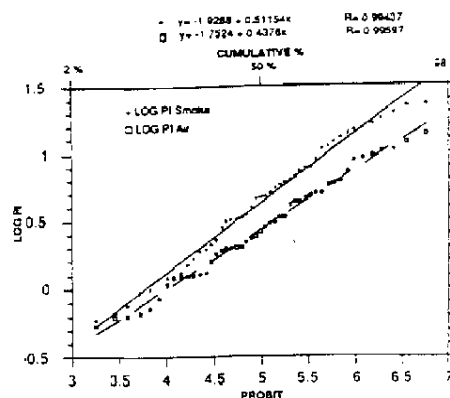


FIG 3. Plot of lognormal distribution of plaque sizes. Plaque indexes are plotted versus probit units (lower abscissa) and cumulative percent of plaque-containing segments (upper abscissa) on log-probability coordinates. For purposes of visual clarity, only plaque indexes corresponding to even integer values of the probability distribution are presented. A probit value of 5 corresponds to the geometric mean of each lognormal distribution. Plus indicates plaque-containing segments from aortas of sidestream smoke-exposed cockerels; and open squares, plaque-containing segments from aortas of air-exposed controls.

Discussion

Cockerel and human arteriosclerotic plaques exhibit similar molecular alterations in addition to their histological and ultrastructural similarities. Serially transmittable dominant transforming elements are present in DNA from both human²⁶ and cockerel²⁷ plaques. We expect that plaque DNA from sidestream smoke-exposed cockerels also would exhibit transforming activity.

In plaques, cell proliferation is episodic, with long stretches of quiescence punctuated by bursts of proliferation (Reference 28 and A. Penn, unpublished observations). Previous findings with cockerels exposed to mainstream smoke²⁰ or to carcinogens^{16,17} argue that the principal effect of these agents is to accelerate development of preexisting arterial plaques. Lung cancer and heart disease data associated with mainstream smoke exposures show that life expectancy for ex-smokers approaches that for "never" smokers as the time since the cessation of smoking increases.²⁹ This suggests that the primary effect of cigarette smoke is more akin to "promotion" than to "initiation." Our previous studies concerning the effects of mainstream smoke exposure on the development of cockerel plaques combined with the results presented here support that contention. In the current study, the anatomic distribution of the plaques (Fig 1), the number of plaques per cockerel, and the number of plaque-containing segments per cockerel (Table 2) were the same for both groups. Thus, sidestream smoke inhalation does not induce formation of new plaques. Rather, the primary effect of sidestream smoke appears to be to make plaques grow at a faster rate, possibly by stimulating proliferation of normally quiescent cells. Some of these quiescent cells may already have a proliferative advantage (ie, are already transformed). Alternatively, sidestream smoke may act first, to com-

plete the transformation of partially transformed cells and then to stimulate them to divide.

It is unlikely that the plaque-stimulating effects of sidestream smoke are due to the transient increases in blood carboxyhemoglobin that result from increased inhalation of carbon monoxide. In cockerels, inhalation of up to 200 ppm carbon monoxide for 2 hours a day, 5 days a week for 16 weeks is without effect on plaque development.³⁰ Here, the 6-hour steady-state chamber carbon monoxide levels were about 35 ppm (Table 1).

The recent report by Zhu et al¹¹ is the only other extensive in vivo study we have found that addresses directly the role of sidestream smoke in plaque development. Despite numerous differences between that study and the one presented here (eg, species, diet, cigarette type, chamber levels of smoke components, plaque measurement techniques, baseline and final plaque levels, and so on), the key finding was essentially the same in both studies. That is, daily inhalation of sidestream smoke for relatively brief periods of time results in striking acceleration of plaque development in experimental animals.

In the studies presented here, white leghorn cockerels were exposed to sidestream smoke for 6 hours a day, 5 days a week, for 16 weeks. This is equivalent to 0.4% of their projected lifespan.³¹ Assuming a human lifespan of 74 or 75 years, an equivalent period of exposure to sidestream smoke would be 3 hours a day for 2.4 years. Additionally, the total sidestream smoke dose achieved in the experimental studies described here is comparable to the dose that can be expected under heavy smoking conditions at home. The predicted total suspended particulates value for environmental tobacco smoke is given by the following equation: $p = nSE/VC$,³² where p is room total suspended particulates (mg/m³), n is number of smokers, S is number of cigarettes per smoker hour, E is total suspended particulates per cigarette (mg), V is room volume (m³), and C is number of air changes per hour. For the experiments we describe, the values were $n=1$, $S=10$, $E=16.5$ mg, $V=1.3$ m³, and $C=14$. The expected value of p was 9.0 and our measured value was 7.93 (average of four chambers, Table 1). In a typical house with one air change per hour and a room measuring 16×10×8 ft (about 36.5 m³), two people smoking a total of five cigarettes of this type per hour would yield $p=2.3$. If the house were a modern one (one third air change per hour), the value of p would triple. In addition, the relatively poor air circulation and low number of air turnovers in the typical house encourage persistence of environmental tobacco smoke even after smoking has stopped.³³ Thus, nonsmokers, including infants and children, living with heavy smokers could be exposed to comparable levels at home. As yet, there are no human data that directly correlate the severity of arteriosclerotic plaques with the levels of exposure to environmental tobacco smoke. Only very recently have data appeared linking environmental tobacco smoke to precancerous lung lesions in man.³⁴ The latter results, those of Zhu et al¹¹ and those presented here, combined with the strong correlation of smoking with both heart disease and lung cancer suggest that individuals exposed to moderate levels of environmental tobacco smoke may be at risk for enhanced arteriosclerotic plaque development.

Acknowledgments

Supported by the Center for Indoor Air Research and NIEHS. We thank Ken Magar and Michael Barbieri for excellent technical assistance and Drs Lung-Chi Chen, Pat Kinney, and Kaz Ito for statistical advice and support.

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